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INHIBITIVE PROPERTIES OF OXIMES
II. THE QUENCHING OF THE FLUORESCENCE OF LUMINOL BY OXIMES

[Following is a translation of an article by Jelka Matković, K. Weber, and Ljerka Palla, of the Institute for Research in Occupational Medicine of the Yugoslav Academy of Sciences and Arts, Zagreb, in the Croato-Serbian periodical Arhiv Higiene Rada (Archives of Occupational Medicine), Vol 14, 1963, pages 95-106.]

In connection with the inhibitive properties of oximes with respect to the luminol reaction in the presence of organophosphoric poisons, measurements are made of the quenching of luminol fluorescence under the influence of various oximes. Numerical values are established for the half-value of such quenching and for quenching constants according to a hyperbolic equation. These values are corrected with regard to secondary effects such as the filter effect on the primary light and the influence of the anion (iodine ion) of certain oximes on luminol. By special experiments it is established that oximes quench the fluorescence of luminol by a dynamic quenching mechanism.

In an earlier work [1] it was established that various oximes definitely inhibit the luminol reaction, i.e. the chemiluminescence induced by the influence of hemiglobin or by potassium (III) hexacyanide. The inhibition manifests itself in a regular diminution of intensity of luminescence with the increase in concentration of the oxime. The results of these experiments are to be accorded toxicological significance, since on the one hand the chemiluminescence of luminol may also be produced by organophosphoric poisons [2], and on the other hand the oximes constitute antidotes in cases of poisoning with organophosphoric pesticides and nerve poisons [3].

For the mechanism of inhibition of the luminol reaction under the influence of oximes, as well as other inhibitive drugs, there are in principle several possibilities. The oximes may act inhibitively on the reaction which gives rise to the charged molecules (ions) of luminol, or may act on the charged molecules themselves. Of course they may also act optically upon the entire system that is luminescing, as internal filters, i.e. media which secondarily absorb optically a part of the light of luminescence. This last is of course

no inhibition at all, but a purely optical effect, which can be separated computationally from the other effects of oximes. With regard to the influence of oximes on the reaction which gives rise to charged molecules of luminol and thus actually occasions chemiluminescence, there are several possible modes of action here, too. Since the more marked chemiluminescence of luminol manifests itself in the presence of complex iron compounds, organophosphoric poisons, or similar substances ("catalysts," promoters), the oximes may form with such compounds inactive combinations and so inhibit the luminol reaction and quench the chemiluminescence. Oximes may also act inhibitively on some other phase of the luminol reaction, whose kinetic mechanism is extraordinarily complex [4] and thus undoubtedly offers excellent opportunities for the influence of any f reign substances that may be present upon the speed with which charged molecules are produced.

In view of these various possibilities of action of oximes on the chemiluminescence of luminol, it was of interest to establish whether oximes are capable of quenching the fluorescence of luminol, and also whether such quenching has a kinetic or a static mechanism [5]. If we find a kinetic mechanism for the quenching of luminol fluorescence, it may be asserted that oximes can work directly on the charged molecules of luminol; but if we find a static mechanism, then it must be concluded that the molecules of luminol and of the oximes may form molecular aggregates (molecular combinations) which lack the property of luminescence. In either case the results of such experiments, which we have carried out and which we shall present here briefly, will give an insight into the mechanism of the inhibitive action of oximes on the luminol reaction, and also into the mechanism of inhibition by oximes in general.

Method of Work

Luminol (3-aminophthalhydrazide) in weak acid solutions shows a pronounced blue fluorescence when illuminated with ultraviolet light. It has been demonstrated [6] that the maximum intensity of fluorescence is in solutions with a $p_H = 4.86$, while solutions whose p_H is less than 3 or more than 7 fluoresce very weakly. We therefore carried out our experiments on the quenching of luminol fluorescence by the influence of oximes in phosphate buffer solution with a $p_H = 4.80$. The luminol concentration in all cases was $2 \cdot 10^{-?}$M [Note: the superscript digit is unreadable in the reproduction from which this translation is made] in the prepared solution which served for measurement purposes. We varied the concentration of the oximes systematically and measured the intensity of fluorescence for various concentrations of oximes. We worked with the following oximes: acetone oxime, acetaldoxime, formaldoxime, the oxime of pyroracemic acid, diacetyl monoxime, isonitrosoacetone, pyridine-4-aldoxime, bromide β(pyridinium-4-aldoxime) methyl

propionate, bromide (pyridinium-4-aldoxime) ethylacetate, 2-pyridine aldoxime iodomethylate, 3-pyridine aldoxime iodomethylate, 1.5-bis (pyridinium aldoxime)-pentane dibromide, 4-pyridine aldoxime iodomethylate, and 1.4-bis (pyridinium aldoxime)-butane dibromide. In addition we also investigated the influence of potassium bromide and iodide on luminol fluorescence, since certain of the oximes mentioned are bromides and iodides respectively.

To determine the mechanism of quenching of fluorescence we used the method of measuring the fluorescence on wet and dry adsorbates on filter paper [7]. For this purpose we also did spectrographic studies of the absorption of various solutions of luminol and oximes, and of solutions with mixtures of these substances.

The intensity of fluorescence of luminol solutions we measured with a photoelectric fluorometer, constructed by combining the spectral photometer of the photoelectric department of the University (made by C. Zeiss, Jena) with a suitable source of ultraviolet radiation (a mercury high-pressure lamp and a suitable combination of optical filters). This apparatus will be made known publicly in another place. With this equipment we were able to determine the extinction coefficients of our substances (luminol and the oximes) in the buffer solution for a wave length of 365 mμ, i.e. for the ultraviolet light used to produce the fluorescence. Since some of the oximes used give weak yellow solutions and so partially absorb the ultraviolet light, it was important in appraising the quenching effect of such oximes to take this action into account computationally as an internal filter effect.

We have expressed the intensity of fluorescence in the presence of oximes throughout in relation (%) to the figure for intensity of the luminol solution without oximes (100%). As a measure of the effectiveness of an oxime as a quenching agent we used the half quenching concentration (c 1/2), which is defined as that molar concentration of the oxime (quenching agent, inhibitor) which reduces the intensity of fluorescence to half (50%). The value for c 1/2 is obtained graphically by interpolation on the curves of quenching of fluorescence.

Results of the Work

First of all it was found that of the oximes tested acetaldoxime and acetone oxime do not quench the fluorescence of luminol, while the other oximes, some of them even in slight concentrations, have a very powerful quenching effect on the fluorescence. The curves in Figure 1 show the dependence of the fluorescence (φ) on the concentration (c) of the oxime of pyroracemic acid (curve 1) and of pyridine-4-aldoxime (curve 2) in solution. It will be seen that the intensity of fluorescence decreases regularly with the increase in concentration of the oxime, and on these curves it is easy to determine the half concentration (c 1/2) in Table 1. Basically similar results (simi-

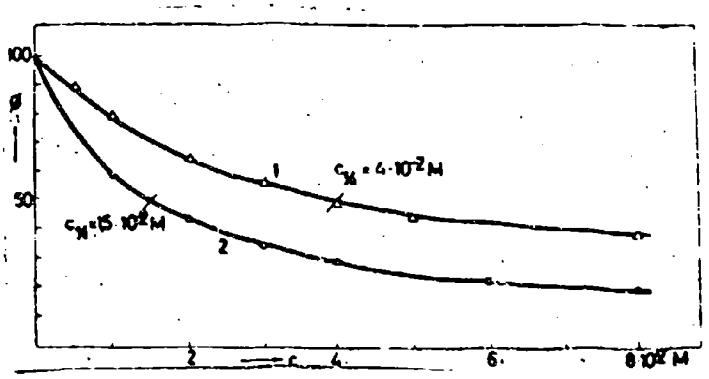

Figure 1. Quenching of the fluorescence of luminol in solutions by the oxime of pyroracemic acid (1) and by pyridine-4-aldoxime (2). ϕ - inteneity of the fluorescence, c - concentration of the oxime.

Table 1. Half concentration (c_K) and constant (A) of quenching of the fluorescence of luminol in solutions (p_H = 4.80).

	Oxime (or halogenide)	$c_K \cdot 10^2$ M	β	β'	$c_K \cdot 10^3$ M
1.	Acetone oxime	--	—	—	—
2.	Acetaldoxime	--	—	—	—
3.	Formaldoxime	280.0	3.572	3.577	296.1
4.	Oxime of pyroracemic acid	40.0	25.00	24.63	40.6
5.	Diacetyl monoxime (DAM)	20.0	50.00	46.50	21.5
6.	Isonitrosoacetone (MINA)	18.5	54.05	43.11	23.2
7.	Pyridine-4-aldoxime (P_4A)	15.0	66.67	63.67	15.7
8.	Bromide β(pyridinium-4-aldoxime) methyl propionate (PAP-β ester)	14.2	70.42	57.79	17.3
9.	Bromide (pyridinium-4-aldoxime) ethyl acetate (PAA-α ester)	14.2	70.42	41.98	23.82
10.	2-pyridine aldoxime iodomethylate (P_2AM)	13.8	72.46	57.43	17.41
11.	3-pyridine aldoxime iodomethylate (P_3AM)	12.8	78.12	72.26	13.84
12.	1.5-bis (pyridinium aldoxime)-pentane dibromide (C-5-dioxime)	11.5	87.10	69.36	14.42
13.	4-pyridine aldoxime iodomethylate (P_4AM)	11.4	87.72	59.54	16.80
14.	1.4-bis (pyridinium aldoxime) butane dibromide (C-4-dioxime)	9.8	102.04	78.67	12.71
15.	Potassium iodide	17.5	57.14	—	—
16.	Potassium bromide	—	—	—	—

larly shaped quenching curves) are also obtained with the other oximes, which are shown with their respective half-concentration values in Table 1. It should be emphasized at once that these half concentrations represent properly speaking the resultant of several influences on fluorescence, which must be distinguished from each other experimentally and computationally.

For this purpose it is necessary first of all to derive the equation of the individual curves of quenching of fluorescence. The dependence of the intensity of fluorescence on the concentration of foreign substances which quench the fluorescence is ordinarily expressed [8] by a hyperbolic equation (the Stern-Volmerov equation) of the following form:

$$\varphi = \varphi_0 \frac{1}{1 + \beta c} \qquad (1)$$

in which φ_0 is the intensity of fluorescence without the presence of foreign substances, φ the intensity in the presence of foreign substances (quenching agents, oximes) in the molar concentration c, and β the quenching constant characteristic of the combination of substances in question. Since this equation does not always interpret quite strictly the course of the quenching curve, in certain cases the exponential equation of quenching is used:

$$\varphi = \varphi_0 \cdot e^{-kc} \qquad (2)$$

(k is the quenching constant according to the exponential equation, e the base of the natural logarithms). In computation, of course, it is also possible to use a combination of the hyperbolic and exponential equations:

$$\varphi = \frac{\varphi_0 \cdot e^{-kc}}{1 + \beta c} \qquad (3)$$

The hyperbolic equation (1) requires a linear relationship between φ_0/φ and c, and in graphic representation the straight line of the equation crosses the coordinate of unity ($\varphi_0/\varphi = 1$). The value of the constant β can be computed from the slope of the line.

In our examples these requirements are met quite well, and it may therefore be considered that for quenching of luminol fluorescence by the action of oximes equation (1) is valid in principle. In no case was any great deviation observed. Figure 2 shows the results of the work, with the above-mentioned manner of representation, for quenching of luminol fluorescence by C-5-dioxime (straight line 1) and isonitrosoacetone (straight line 2). We obtained similar results with other oximes. The values for the constant β obtained from the slope of the line are presented in Table 1.

Oximes in aqueous solutions are usually more or less yellow in color. In view of the dissociation in tautomeric oximes in equilibrium [1], this color is more pronounced in alkaline and less so in acid solutions. It is definitely

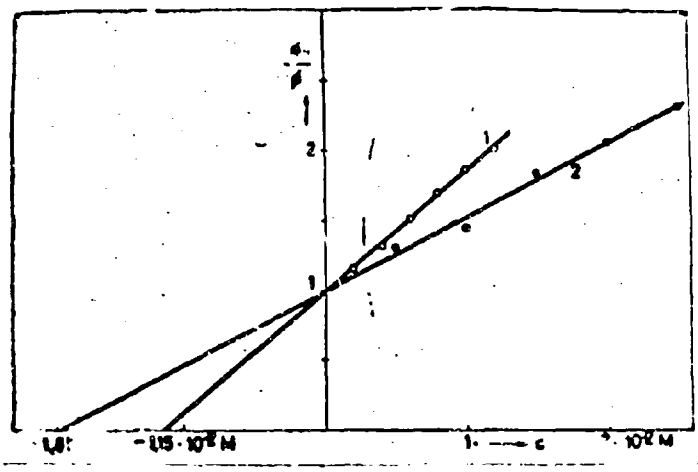

Figure 2. The dependence φ_0/φ on the concentration of the oxime (c) for 1.5-bis (pyridinium aldoxime)-pentane dibromide (line 1) and for isonitrosoacetone (2).

present at least at p_H = 4.8. In connection with the experiments described here this means that the oximes will certainly absorb a part of the ultraviolet light that produces the fluorescence. In consequence of this a certain (minor) part of the quenching of fluorescence will be attributable to the action of the oxime as an "internal" filter of the ultraviolet light. Such a filter effect is not a "true" inhibitive action, but an (undesirable) optical effect, which should be separated computationally from the inhibition itself. In order to do this it was necessary to establish experimentally the extinction coefficient (ε) of all the oximes and also of luminol for the light (λ = 365 mμ) which was used to produce the fluorescence.

Determination of the extinction coefficient for the light in question was accomplished by fluorometric measurements of the absorption of the solution of the oximes and luminol, and by means of the fluorometer already mentioned. The results of these measurements are presented in Table 2. The value of the decimal molar extinction coefficient (ε) was worked out for the various concentrations of oximes, but strictly constant values were not always obtained. This is obviously connected with the above-mentioned equilibrium of these substances in aqueous solutions. With regard to that the values for ε in Table 2 that are marked with an asterisk (*) refer to the half concentration for quenching of fluorescence by the oximes in question, while the values without that sign are valid for a greater range of concentration around the halfway mark.

From the data in the table it will be noted first of all that luminol absorbs ultraviolet light with a wave length of

Table 2. Extinction coefficient (ε for λ = 365 mμ) and absorption per unit in mixtures with luminol at the half concentration for quenching of fluorescence.

	Oxime (c. luminol)	ε	A_2 in %
1.	Formaldoxime	0.045	2.73
2.	Oxime of pyroracemic acid	0.085	0.745
3.	Pyridine-4-aldoxime (P_4A)	0.682	2.22
4.	Diacetyl monoxime (DAM)	0.809	3.48
5.	3-pyridine aldoxime iodomethylate (P_3AM)	1.500*	4.11
6.	Isonitrosoacetone (MINA)	2.635	10.13
7.	Bromide β(pyridinium-4-aldoxime) methyl propionate (PAP-β ester)	3.005*	8.92
8.	2-pyridine aldoxime iodomethylate (P_2AM)	3.623*	10.37
9.	1.5-bis (pyridinium aldoxime)-pentane dibromide (C-5-dioxime)	4.238	10.12
10.	1.4-bis (pyridinium aldoxime)-butane dibromide (C-4-dioxime)	5.920	11.95
11.	4-pyridine aldoxime iodomethylate (P_4AM)	7.017*	16.06
12.	Bromide (pyridium-4-aldoxime) ethylacetate (PAA-α ester)	7.558*	20.91
13.	Luminol	2102.06	--

365 mμ incomparably more effectively than any of the oximes tested. Consequently the action of the oximes as internal filters will be insignificant in the conduct of experiments with the quenching of fluorescence of luminol, and the corrections for that effect proportionally small. The absorption of the oximes in mixture with luminol in solution (A_2 in %) is computed according to the following equation [8]:

$$A_2 = 100\frac{\varepsilon_2 c_2}{\varepsilon_1 c_1 + \varepsilon_2 c_2}\left[1 - 10^{-(\varepsilon_1 c_1 p + \varepsilon_2 c_2 p)}\right] \qquad (4)$$

In the above equation ε_1 and ε_2 are the extinction coefficients of luminol and of the oxime, c_1 and c_2 are the corresponding concentrations of these substances, and p the thickness of the layer in the experiment. For the half concentration for quenching fluorescence we have worked out values for A_2 for all the oximes and entered them in Table 2. It will be seen that the per unit absorption of ultraviolet light in the oximes amounts in the extreme case, for PAA-α_A^{oxime} to only about 20%, while about 80% of the light is absorbed by luminol. For the other oximes A_2 has smaller, in some cases much smaller, values.

In conducting experiments on the quenching of fluorescence it is possible to take the apsorption of ultraviolet light in molecules of oximes into account computationally by modifying equation (1) to read as follows:

$$\varphi = \frac{\varphi_0 - A_2}{1 + \beta' c} \qquad (1a)$$

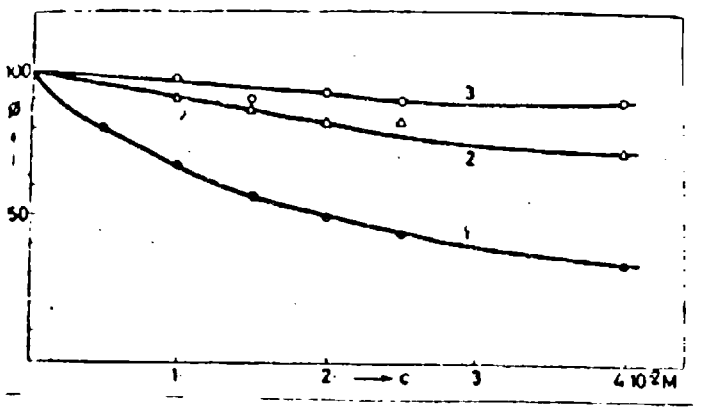

Figure 3. Quenching of the fluorescence of luminol by diacetyl monoxime in solutions (1), on moist paper (2), and on dry paper (3). Φ - intensity of fluorescence, c - concentration of the oxime.

Table 3. Corrected constant (β''_1) of quenching of luminol fluorescence by cation oximes and corresponding half concentrations (c" 1/2).

	Oxime	β''_1	c"1/2
1.	2-pyridine aldoxime iodomethylate (P_2AM)	0.61	1.64
2.	3-pyridine aldoxime iodomethylate (P_3AM)	15.44	0.065
3.	4-pyridine aldoxime iodomethylate (P_4AM)	2.74	0.365

In the above equation β' is the constant of quenching of fluorescence which has been corrected to take account of the absorption of light in the molecules of the oxime. If the computation is done with the half concentration of quenching of fluorescence, the following equation will hold:

$$\beta' = \frac{\frac{100 - A_2}{50} - 1}{c_{1/2}} \qquad (1b)$$

The values for the corrected quenching constant (β') are also entered in Table 1. It will be seen that some of these corrections are really insignificant, but that they have some significance for the quenching of luminol fluorescence by the following oximes: PAP-β ester, PAA-α ester, P_2AM, P_4AM, C-5-dioxime, and C-4-dioxime. According to equation (1) the reciprocal value of the constant gives the value of the half concentration ($c_{1/2}$) of quenching of fluorescence, corrected to take account of the absorption of ultraviolet light. The values of this quantity are also entered in Table 1.

The corrected values for the half concentration of the various oximes represent for most of the oximes tested the

true values for quenching of fluorescence. That is to say that these values correspond to the influence directly exerted by the oxime on the charged molecules of luminol. That, however, is not the case with those oximes which according to their chemical constitution constitute iodides. It may be conjectured that such oximes in aqueous solutions give off an anion of iodine along with a cation of the oxime. Now such an anion also quenches the fluorescence of luminol, with a half concentration which is shown (for KI) in Table 1, and it is believed that the cation of potassium does not influence fluorescence. The quenching of fluorescence by such "oximic iodides" is in any case a complex phenomenon which has three components: 1) quenching in consequence of absorption of primary light (A_2), 2) quenching in consequence of action of the oximic cation on charged molecules of luminol, and 3) quenching in consequence of action of the iodine anion on charged molecules of luminol. The first effect we have already corrected for computationally by equations (1a) and (1b), and the second and third may be separated from each other by a quenching equation which applies to systems which contain two foreign substances (quenching agents), and which will obviously have the following form:

$$\varphi = \frac{\varphi_0 - A_2}{1 + \beta_1'' c_1 + \beta_2 c_2} \qquad (5)$$

In our case β_1'' is the corrected (unknown) constant of quenching by the iodide (see Table 1), and $c_1 = c_2$ (the concentration of oximic iodide). It is evident that by equation (5) we can easily compute the value β_1'' for any oximic cation. For the half concentration of quenching we have the equation:

$$\beta' = \beta_1'' + \beta_2 = \frac{\frac{100 - A_2}{50} - 1}{c_{1/2}} \qquad (5a)$$

and also

$$\beta_1'' = \beta' - \beta_2 \qquad (5b)$$

The values of the constants of quenching of luminol fluorescence by only the oximic cations of certain iodides are presented in Table 3. That table also gives the half concentration of quenching by the same phenomenon (reciprocal values of the corresponding constants).

It is obvious that the cations of these oximes have a relatively very slight effect on the charged molecules of luminol. The principal effect in these cases is to be attributed to the iodine anion. But the specific property is to be attributed to the cation of P3AM, however, that it markedly quenches the luminescence of luminol.

For determining the mechanism of the quenching of fluorescence by the addition of foreign substances there are several possibilities, such as (9) determining the influence of temperature on the quenching, measuring the absorption spectra of

fluorescent substances in the absence and in the presence of quenching agents, determining the intensity of fluorescence of adsorbates on filter paper, and measuring the degree of polarization of the fluorescence in the absence and in the presence of quenching agents. Of the methods mentioned, we used chiefly the study of absorption spectra of luminol solutions. By spectrographic photographs of various objects we demonstrated that the absorption spectrum of luminol in solution at $p_H = 4.8$ is not modified by the addition of any of the oximes mentioned in Table 1. This result speaks in favor of the supposition that under the experimental conditions mentioned the oximes do not form molecular associations or aggregates with luminol, but quench the luminescence of luminol by a kinetic mechanism.

In order to confirm this result with certainty, we made parallel measurements of the intensity of fluorescence of luminol in the presence of the above-mentioned oximes with solutions and with moist and dry adsorbates on filter paper. According to earlier hypotheses [7], a static mechanism of quenching of fluorescence would account for quenching curves for solutions and adsorbates that did not differ essentially from each other, while a kinetic mechansim of quenching would call for a significant decrease in quenching when the object that fluoresces is bound by adsorption to paper. In specific cases we did in fact regularly obtain quenching curves for adsorbates that differed essentially from the corresponding curves of the solutions, and moreover the quenching was significantly diminished by the process of adsorption and by the drying of such adsorbates. The curves of Figure 3 illustrate this phenomenon for the quenching of luminol fluorescence by diacetyl monoxime. We obtained fundamentally similar results for quenching by most of the other oximes. Only in the quenching of luminol fluorescence with C-4 and C-5-dioxides was there no such great and pronounced difference in the quenching curves in the dissolved and the adsorbed states. Differences did exist, however, and it could not be concluded that in these cases there was a static mechanism.

In general it was possible to conclude that the oximes quench the fluorescence of luminol by a kinetic mechanism. This means that molecules or ions of the oximes can have an effect on the charged molecules of luminol while the excited state exists, and as a result of this kinetic effect the energy of excitation degenerates into heat, and the luminol molecule then passes out of the charged state into the normal state without the emission of the light of luminescence.

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Summary [printed in German]

Since it had been established in an earlier article (1) that various oximes are capable of inhibiting the chemiluminescence of luminol (3-aminophthalhydrazide) which has been induced by complex iron compounds or by poisons of the organophosphoric group of compounds, it was now of interest to determine whether the oximes also quench the fluorescence of luminol. In this direction experiments were done with luminol solutions in a phosphate buffer (p_H = 4.8) and with various oximes, which are listed in Table 1. The $c_{1/2}$ values in that table give the half-value concentration of the solution (the molar concentration of the oxime which quenches the fluorescence down to one half). Figure 1 shows the quenching curves for two oximes by way of example. The β values in Table 1 are the values of the quenching constants according to hyperbolic equation 1. That this equation actually holds is evident from the direction of the straight lines of the graph in Figure 2, which were also used in computing the β values.

Since in some cases the oximes in solutions are yellow in color and so absorb the primary ultraviolet light in the fluorescence experiments, it was necessary to take account of this internal filter effect experimentally and computationally. For that reason the values of the molar extinction coefficients (ϵ) of the oximes and of luminol for λ = 365 mμ were determined fluorometrically (Table 2). From the values of the extinction coefficients the specific absorptions of the oximes (A_2) in the mixture with luminol in the

solutions corresponding to the half-value concentrations were computed according to equation (4). By means of the values obtained, the half-value concentrations and the quenching constants could be corrected for the internal filter effect (equations 1a and 1b; $c'_{1/2}$ and Λ' in Table 1). But in a few cases the effect of the anion (iodine ion) also had to be considered. This was done by means of equations (5), (5a), and (5b). The thus corrected values Λ'' and $c''_{1/2}$ for the cations of the oximic iodides are shown in Table 3.

By measurements of the light absorption of luminol solutions in the presence and in the absence of oximes, and also by measurements of the quenching curves of adsorbates on filter paper (7) it was established (curves of Figure 3) that the quenching of luminol fluorescence by oximes is to be attributed to a dynamic quenching mechanism.

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